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Intracellular granules in several types of leukocytes contain preformed proteins whose secretions contribute to immune and inflammatory functions of leukocytes, including eosinophils, cells notably associated with asthma, allergic inflammation, and helminthic infections. Cytokines and chemokines typically elicit extracellular secretion of granule proteins by engaging receptors expressed externally on the plasma membranes of cells, including eosinophils. Eosinophil granules, in addition to being intracellular organelles, are found as intact membrane-bound structures extracellularly in tissue sites of eosinophil-associated diseases. Neither the secretory capacities of cell-free eosinophil granules nor the presence of functional cytokine and chemokine receptors on membranes of leukocyte granules have been recognized. Here, we show that granules of human eosinophils express membrane receptors for a cytokine, IFN-γ, and G protein–coupled membrane receptors for a chemokine, eotaxin, and that these receptors function by activating signal-transducing pathways within granules to elicit secretion from within granules. Capacities of intracellular granule organelles to function autonomously outside of eosinophils as independent, ligand-responsive, secretion-competent structures constitute a novel postcytolytic mechanism for regulated secretion of eosinophil granule proteins that may contribute to eosinophil-mediated inflammation and immunomodulation.

allergy | specific | eotaxin | IFN-γ

Human eosinophils, leukocytes notably associated with allergic, anthelminthic parasite, and other immune responses (1–3), contain an abundance of large cytoplasmic granules ultrastructurally unique because of their internal crystalline cores. As intracellular organelles, these granules are central to the functional responses of eosinophils in that granules house preformed stores of (*i*) four major cationic proteins, including eosinophil cationic protein (ECP), major basic protein (MBP), and eosinophil peroxidase (EPO); (*ii*) hydrolytic enzymes; and (*iii*) more than 30 cytokines, chemokines, and growth factors (3, 4). The extracellular release of these diverse granule proteins may occur, at times, by exocytosis mediated by fusion of granules with the plasma membrane, a process mobilizing the entire protein repertoire of granules. Alternatively, eosinophil secretion more often involves an intracellular process of piecemeal degranulation, whereby granule proteins are mobilized and transported in secretory vesicles for extracellular release from intact eosinophils (4). Piecemeal degranulation results in the selective secretion of granule-derived proteins and is initiated by ligands (e.g., IFN-γ) (5) and eotaxin (also called CCL11) (6), acting via their cognate plasma membrane-expressed receptors. A third, as yet, enigmatic mode of eosinophil degranulation arises from cytolysis of eosinophils that deposits intact membrane-bound eosinophil granules extracellularly (7). With their unique ultrastructure, free extracellular eosinophil granules have been recognized in the airways or tissues in association with diverse disorders, including allergic asthma and rhinitis, dermatitis, helminth infections, eosinophilic esophagitis, and urticaria (7–12). Neither functional roles nor consequences of cell-free extracellular eosinophil granules have been delineated. Here, we evaluated the capacity of eosinophil granules to function extracellularly as secretory organelles. These investigations were engendered by recent findings. First, intracellular eosinophil granules contain substantial quantities of chemokine and cytokine receptors (13). Second, upon eosinophil stimulation, vesicles arise, in a brefeldin A (BFA)–inhibitable process, from tubular membranes within granules transporting granule cytokines and cationic proteins (13–15). We found that cell-free eosinophil granules respond to IFN-γ and eotaxin stimuli via cognate granule membrane-expressed receptors, topologically oriented to respond to cytokine and chemokine proteins acting externally upon granule membranes. Both IFN-γ and eotaxin activate intragranular signaling pathways and BFA-inhibitable secretory mechanisms to elicit secretion of granule-derived enzymes, cytokines, and cationic proteins.

Results

Subcellular Isolation and Characterization of Purified Eosinophil Granules. We studied the secretory responses of cell-free granules isolated by density gradient subcellular fractionation from human eosinophils, finding that denser granule-enriched fractions were distinct from buoyant secretory vesicles [supporting information (SI) Fig. S1*A*]. By flow cytometry, isolated granules contained immunoreactive MBP following membrane permeabilization (Fig. S1*B*) and were devoid of contaminating plasma membrane MHC class I protein expression (Fig. S1*C*). By transmission electron microscopy (TEM), purified granules exhibited intact surrounding membranes (Fig. S1*D*). Isolated granules expressed lysosome-associated membrane proteins (LAMP)-2 (Fig. S1*E*) and -3 (CD63) (Fig. S1*F*) previously localized to eosinophil granules (16, 17). Like granules in intact eosinophils (Fig. S1*Gi*), isolated granules stained with lysosomotropic acridine orange (AO) (Fig. S1*Gii*).

Eosinophil Granules Function as Cell-Free Secretion-Competent Organelles in Response to IFN-γ and Eotaxin. The presence of free extracellular eosinophil granules has been recognized *in vivo* in tissues in association with diverse eosinophilic disorders (Fig. 1; refs. 7–12), but potential functional roles of their deposition have not been delineated. Granules isolated by subcellular fraction-

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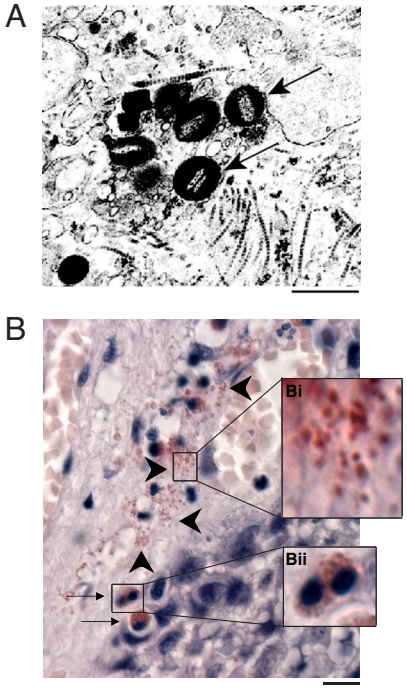

Fig. 1. Extracellular eosinophil granules are recognized in tissues in association with diverse disorders. (*A*) Extracellular eosinophil granules (arrows) are present in a skin lesion of a subject with hypereosinophilic syndrome. (Scale bar: 1 μm.) (*B*) Cell-free eosinophil granules (arrowhead and higher magnified in *Bi*) and intact eosinophils (arrows and higher magnified in *Bii*) were identified in a skin biopsy of a patient with verrucous cancer. Preparations were stained with H&E and examined by light microscopy. [Scale bar: 17 μm, 3.6 μm (*Bi*), and 6 μm (*Bii*).]

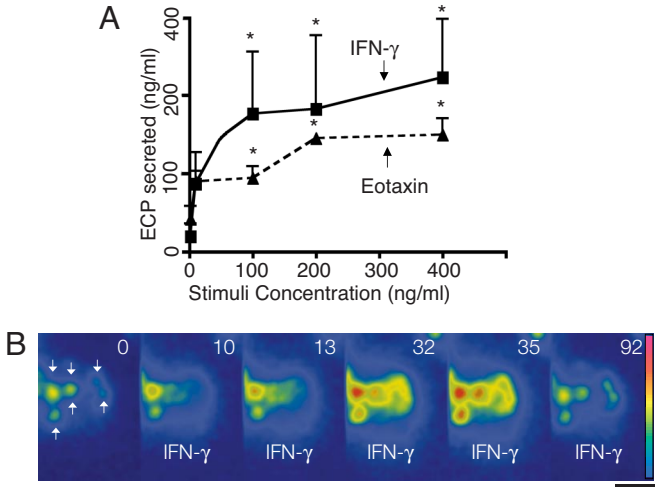

Fig. 2. Extracellular eosinophil granules function as cell-free secretion-competent organelles in response to IFN-γ and eotaxin. (*A*) Eosinophil granules secrete ECP in response to IFN-γ (solid line and squares) and eotaxin (dashed line and solid triangles). Secreted ECP levels, means of duplicates $\pm$ SD, are ECP levels from stimulated granules minus ECP levels from unstimulated granules, as assayed after 1 h by ELISA. Data from one experiment are representative of three, each of which demonstrated significant dose-dependent secretion elicited by eotaxin and IFN-γ (by ANOVA). *$P < 0.05$ vs. IFN 1-ng/ml stimulated samples. (*B*) AO loaded granules (arrows) respond 0, 10, 13, 32, 35, and 92 sec after IFN-γ stimulation with intense transient fluorescent flashes indicative of release of monomeric AO. Fluorescence intensity is pseudocolored, with red representing the greatest intensity as indicated by the scale color. (Scale bar: 3 μm.)

ation, in response to stimulation with the cytokine IFN-γ and the chemokine eotaxin, dose dependently released ECP (Fig. 2*A*). Secretion of ≈0.5%–2% (m/m) of granule ECP (Fig. S2*A*) was rapid, starting within 5 min and reaching plateau values by 30 min (Fig. S2*B*). Likewise, both stimuli elicited secretion of granule-derived β-hexosaminidase and EPO (Fig. S2*C*). The release of sequestered AO from organelles generates a fluorescent green "flash" attributable to release of monomeric AO at neutral pH (18). To ascertain that isolated granules were the organelles responsive to agonists, we demonstrated that AO-loaded granules, in response to IFN-γ and eotaxin stimulation (Fig. 2*B*, Movie S1, and Movie S2), exhibited activation, as evidenced by intense fluorescent flashes attributable to granule release of AO.

To assess whether IFN-γ and eotaxin acted via granule membrane-expressed receptors, we analyzed by flow cytometry the expression and topology of receptor proteins for these two agonists. Cell-free granules without membrane permeabilization exhibited staining for IFN-γ receptors (IFN-γR) using a mAb specific for the "extracellular" region of the IFN-γR α chain (Fig. 3*A*). Likewise, Ab to the extracellular ligand-binding region of the G protein–coupled CCR3 receptor for eotaxin (Fig. 3*B*) detected CCR3 expression on isolated granules, whereas staining with a polyclonal Ab (pAb) to the "intracellular" carboxy-terminal domain of CCR3 was negative (Fig. 3*C*) on nonpermeabilized granules. To confirm that the association of CCR3 with isolated granules was not an artifact of subcellular fractionation, eosinophils were incubated with plasma-coated Sepharose beads, an interaction that elicits eosinophil activation and cytolytic granule release (19). Granules cytolytically extruded from eosinophils likewise expressed extracellular domains of CCR3 on granule membranes (Fig. S3) and, following AO loading, responded to eotaxin stimulation with intense fluorescent flashes of released AO (Movie S3). Thus, granule membranes expressed two structurally distinct receptors, the heterodimeric IFN-γR and the heptahelical G protein–coupled receptor (GPCR) CCR3, both of which exhibited external ligand-binding orientations typical of extracellular domains, despite arising from an intracellular cytosolic milieu.

IFN-γ– and Eotaxin-Elicited ECP Secretion Is Mediated by Intragranule Signaling Pathways. We evaluated signaling pathways within cell-free eosinophil granules that coupled IFN-γR– and CCR3-initiated signaling to granule secretion. Genistein, a tyrosine kinase inhibitor, dose dependently inhibited ECP release from IFN-γ–stimulated granules (Fig. 4*A*). IFN-γ–induced ECP release was also dose dependently inhibited by SB203580 and SB202190, both p38 MAPK inhibitors (Fig. 4*B*), and by calphostin C, a likely PKC blocker (Fig. 4*C*). In contrast, LY2924002, a PI3K inhibitor, did not inhibit IFN-γ–stimulated ECP secretion from granules (Fig. 4*D*). Likewise, eotaxin-initiated signaling within isolated granules was evaluated. Pertussis toxin, an inhibitor of $G_{i/o}$ type G proteins, dose dependently inhibited eotaxin-elicited secretion of ECP (Fig. 4*E*), compatible with eotaxin acting via its $G_{i/o}$ protein-coupled CCR3, GPCR. The p38 MAPK inhibitors, SB203580 and SB202190 (Fig. 4*F*), the PKC inhibitor, calphostin C (Fig. 4*G*), and the PI3K inhibitor, LY2924002 (Fig. 4*H*), dose dependently inhibited eotaxin-stimulated ECP secretion from eosinophil granules. Moreover, isolated granules contained signaling PKC and p38 MAPK (Fig. 4*I*). Eotaxin stimulation of granules elicited the activation-associated phosphorylation of granule and eosinophil p38 MAPK (Fig. 4*J*). Furthermore IFN-γ stimulation of granules induced phosphorylation of tyrosine residues in a protein with the predicted molecular weight for the IFN- γR α chain. The phosphorylation was inhibited by preincubation of granules with

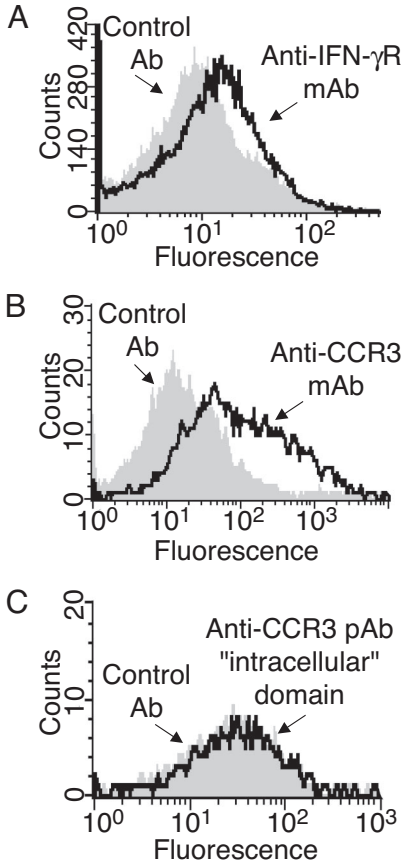

Fig. 3. Extracellular eosinophil granules express IFN-γ and CCR3 receptors on their membranes. By flow cytometry of isolated non–membrane-permeabilized granules, granule surface membranes expressed extracellular domains of the IFN-γR α chain (*A*) and the CCR3 receptor for eotaxin (*B*) but not the nonligand (carboxy)-terminal intracellular domain of CCR3 (*C*). Shaded histograms and solid lines represent staining with control Ab and mAb or pAb-specific Abs, respectively. Data are from one experiment, representative of three.

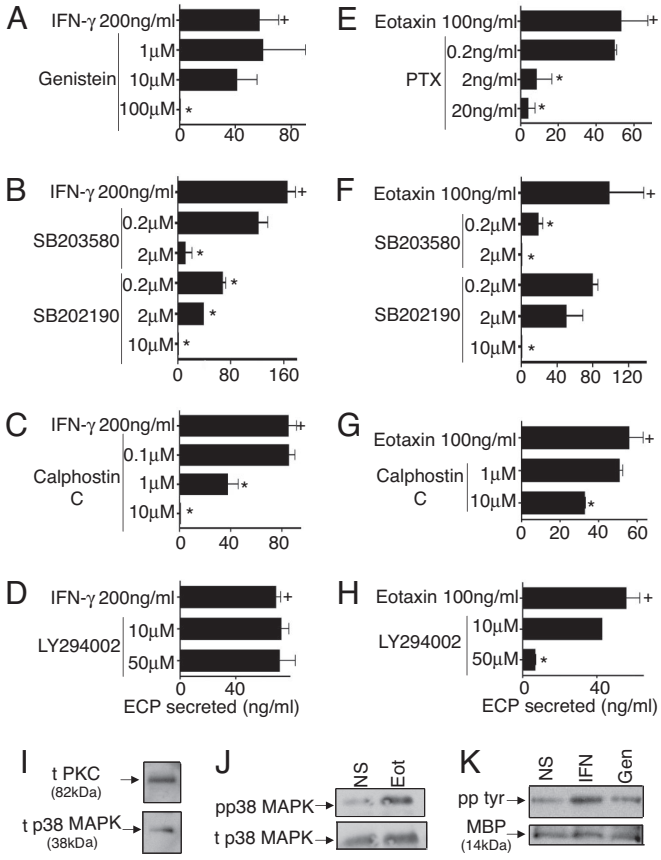

Fig. 4. IFN-γ– and eotaxin-elicited ECP secretion by cell-free granules is mediated by intragranular signaling pathways. Pretreatment of eosinophil isolated granules with indicated concentrations of genistein, a tyrosine kinase inhibitor (*A*), SB203580 and SB 202190, both p38 MAPK inhibitors (*B*), and calphostin C, a PKC inhibitor (*C*), with each inhibited ECP secretion elicited by IFN-γ. (*D*) PI3K inhibitor, LY294002, did not inhibit ECP secretion elicited by IFN-γ. Eotaxin-induced ECP secretion was inhibited by pertussis toxin (PTX) (*E*), SB203580 and SB 202190 (*F*), calphostin C (*G*), and LY294002 (*H*). Eosinophil granules were pretreated with inhibitors for 15 min and stimulated with IFN-γ (200 ng/ml) or eotaxin (100 ng/ml) for 1 h. Secreted ECP levels, means of duplicates ± SD, are ECP levels from stimulated granules minus ECP levels from unstimulated granules as assayed by ELISA. Data, one experiment representative of three, were analyzed by one-way ANOVA, followed by the Newman-Keuls test. + and * represent $P < 0.05$ compared with nonstimulated and cytokine/chemokine-stimulated granules, respectively. (*I*) Isolated granules contain PKC and *p*-38 MAPK. (*J*) Eotaxin (100 ng/ml, 1 min) induced phosphorylation of *p*-38 MAPK on granules. t, total; p, phosphorylated; NS, not stimulated; Eot, eotaxin-stimulated. (*K*) IFN-γ (200 ng/ml, 5 min) induced tyrosine phosphorylation of a granule protein, and genistein (100 μM) inhibited phosphorylation. Loading controls were blotted for granule MPB. pp tyr, phosphorylated tyrosine residues; NS, not stimulated; IFN, IFN-γ–stimulated; Gen, genistein.

genistein (Fig. 4*K*). These findings provide direct evidence that granule membrane receptors are functional and able to initiate signaling. Thus, although the two receptor-initiated secretory responses of cell-free eosinophil granules differed in their reliance on PI3K-mediated signaling, both granule secretory responses were dependent on specific signaling transducing pathways within granules.

BFA Inhibits Secretion from IFN-γ– and Eotaxin-Stimulated Eosinophil Granules. We previously demonstrated that intracellular eosinophil granules contain membranous vesiculotubular networks that contribute to forming secretory vesicles within granules (14, 15). BFA inhibits vesicular transport-mediated piecemeal degranulation by eosinophils; and one site of BFA's inhibitory action is within granules, as evidenced by BFA inducing the collapse of intragranular vesiculotubular membranes into membranolipid deposits recognized by TEM (14). We evaluated whether BFA had similar effects on stimulated secretion from isolated eosinophil granules. BFA dose dependently inhibited IFN-γ– (Fig. 5*A*) and eotaxin- (Fig. 5*B*) elicited ECP secretion and inhibited eotaxin-elicited β-hex (Fig. S4) secretion from cell-free granules. Moreover, as assessed by TEM, BFA treatment of isolated IFN-γ–stimulated granules increased numbers of intragranule membranolipid deposits (Fig. 5*C*) (11.0 deposits per 100 granules with BFA vs. 2.6 deposits per 100 granules without BFA), analogous to BFA's antisecretory effects on granules within eosinophils. These findings suggest that the agonist-receptor–elicited secretion of eosinophil granule proteins is likely dependent on the membranotubular network within granules probably involved in the mobilization and secretion of their contents (13–15).

Isolated Eosinophil Granules Secrete Cytokines upon IFN-γ Stimulation. In addition to cationic proteins (e.g., ECP) and hydrolases (e.g., β-hex), eosinophil granules contain diverse cytokines, chemokines, and growth factors. Differential regulated secretion of preformed stores of these mediator proteins contributes to the capacity of eosinophils to modulate immune and other host

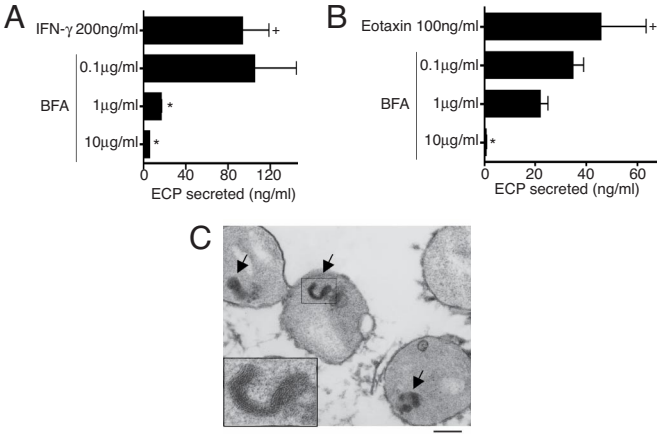

Fig. 5. BFA inhibits ECP secretion from IFN-γ– (*A*) and eotaxin- (*B*) stimulated eosinophil granules. Secreted ECP levels, means of duplicates ± SD, are ECP levels from stimulated granules minus ECP levels from unstimulated granules, as assayed after 1 h by ELISA. Data, one experiment representative of three, were analyzed by one-way ANOVA, followed by the Newman-Keuls test. + and * represent $P < 0.05$ compared with nonstimulated and cytokine/chemokine-stimulated granules, respectively. (*C*) BFA promotes the collapse of membranotubular networks within granules forming electron-dense membranolipid deposits (arrows, inset). (Scale bar: 200 nm.) Data are from one experiment, representative of three.

responses (4). As for granules within intact eosinophils, extracellular eosinophil granules can be sources of secreted eosinophil-derived cytokines. IFN-γ dose dependently stimulated secretion of IL-4 and IL-6 but not IL-13 (Fig. 6) and not IL-8, IL-10, or IL-12 (p70) from isolated granules. Thus, agonist-dependent processes mediating eosinophil cytokine secretion by cell-free granules can be selective, as demonstrated with intact eosinophils (4, 20, 21), providing novel insights into the potential regulated capabilities of eosinophil granules, when released extracellularly, to be variably responsive to cytokine, chemokine, and other agonists in differentially secreting their granule-stored proteins.

Discussion

Although eosinophils, like other granule-bearing leukocytes, have been recognized for their capacities to release granule-derived proteins from within leukocytes, our findings are notable for several reasons. First, cytoplasmic granules of eosinophils, the quantitatively predominant and distinguishing intracellular organelles within these leukocytes, have evolved in a fashion that uniquely enables these organelles also to function extracellularly as secretion-competent structures responsive to receptor-mediated agonists. Granules not only express functional receptors on their surface membranes but couple these receptors to intragranular signaling and intragranular membranotubular network-based secretion responses.

Second, the topology of receptors expressed on granule membranes, by which nominally extracellular ligand-binding receptor domains are displayed externally on granule membranes, raises the possibility that such receptors expressed on intracellular granules might be indicative of roles of cytokines and chemokines as intracellular regulators of granule secretion. Widely abundant GPCRs have traditionally been considered to transduce signals at the plasma membrane (22) and, more recently, at nuclear membranes (23). Although a cell-permeant lipid, estrogen, has signaled through an endoplasmic reticulum–based GPCR (24), other nonnuclear intracellular organellar sites of localization and function for GPCRs have not been identified. In response to extracellular eotaxin, which is not cell permeable, CCR3 undergoes ligand-induced internalization from the eosinophil plasma membrane (25). That CCR3 might mediate ligand-initiated signaling intracellularly has not been suggested, although we previously documented CCR3 protein localization at isolated eosinophil granules (13). Our current findings recognize an intracellular localization for a functional GPCR, CCR3, on eosinophil granule membranes. Limited prior experiments indicated that IFN-γ can signal intracellularly, but sites of such signaling, other than potentially at the nucleus for IFN-γ (26) after receptor-mediated endocytosis, have not been identified. Thus, localization of functional IFN-γR to eosinophil granule membranes identifies a subcellular localization for this ligand-responsive receptor. Recognition that structurally distinct heterodimeric IFN-γRs and heptahelical CCR3 GPCRs are expressed on eosinophil granule membranes and are functional in mediating cytokine- and chemokine-elicited granule-derived secretion, extracellularly and potentially intracellularly, extends our understanding of the function of these receptors on the membranes of intracellular organelles.

Finally, although cytolytic release of intact eosinophil granules into tissues is well documented (7–12) but of heretofore ill-understood consequence, the responsiveness of cytolytically released, extracellular, fully secretion-competent granules expands the capacities of eosinophils to contribute to modulating host and immune responses (1, 2, 27). Because eosinophil granules have ultrastructurally unique cores and distinct cationic protein content that can be detected extracellularly by EM, light microscopy, and immunofluorescence microscopy (7–9, 28), there may be a detection bias in recognizing extracellular granules of eosinophils in comparison to granules of other leukocytes. Intact extracellular granules from other leukocytes have rarely been sought. In one study that enumerated both free eosinophil and neutrophil granules, free anti-elastase–staining neutrophil granules were found in tissues of subjects with chronic rhinosinusitis but much less commonly than free MBP-positive eosinophil granules (28). Isolated mast cell granules also have exhibited ligand-elicited ion fluxes (29). Whether granules of other leukocytes may likewise express chemokine or cytokine receptors has not been evaluated, although CCR3 has been demonstrated on intracellular mast cell granules (30) and IL-10 receptors on neutrophil granules (31). Potential capacities of granules from other leukocytic cells (e.g., neutrophils, mast cells, NK cells) to likewise function extracellularly remain to be assessed. Nevertheless, for eosinophils that undergo cytolysis and granule extrusion in tissues, the liberated extracellular

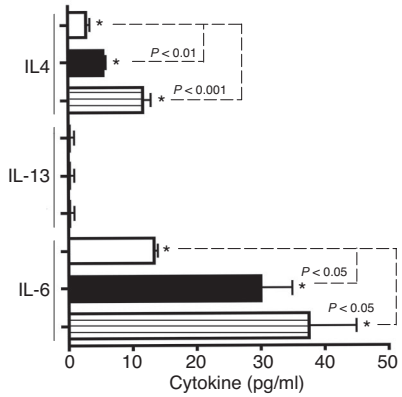

Fig. 6. Isolated eosinophil granule cytokine secretion upon IFN-γ stimulation. IFN-γ dose dependently induced release of IL-4 and IL-6 but not IL-13 after 1 h. Cytokine levels in unstimulated samples were undetectable. Open, closed, and hatched columns represent stimulation with 100, 200, and 400 ng/ml IFN-γ, respectively. Data represent the means of duplicates ± SD. Results, one experiment representative of three, were analyzed by one-way ANOVA, followed by the Newman-Keuls test. *$P < 0.05$ compared with nonstimulated granules.

granules, with their rich content of preformed cytokines and cationic proteins, may function as cluster-bombs in continuing to exhibit "postmortem" agonist-specific and differential secretion of eosinophil granule-derived proteins.

Methods

Eosinophil Purification and Subcellular Fractionation. Eosinophils were obtained from the blood of healthy and atopic donors by previously described methods (13) with modifications that included collecting blood with pH neutral sodium citrate buffer (100 mM, pH 7.4) and not using erythrocyte lysis. Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. For subcellular fractionation, eosinophils ($10–30 \times 10^6$) were resuspended in disrupting buffer (5) supplemented with 5 μg/ml DTT and subjected to nitrogen cavitation (Parr) (600 psi, 10 min). Postnuclear supernatants, recovered after centrifugation ($400 \times g$, 10 min), were ultracentrifuged ($100,000 \times g$, 1 h at 4 °C) in linear isotonic Optiprep (Axis-Shield) gradients (0%–45% in disrupting buffer without protease inhibitors).

Stimulation of Isolated Eosinophil Granules. Subcellular fractions containing isolated granules were mixed with RPMI plus 0.1% OVA (Sigma), followed by centrifugation ($2,500 \times g$, 10 min). Pelleted granules, resuspended in 250 μl of the same medium, were incubated with IFN-γ (Biosource International) or eotaxin (R&D Systems). Treatments with BFA (Biomol), calphostin C (Sigma), genistein (Sigma), LY294002 (Biomol), SB203580 (Calbiochem), SB202190 (Calbiochem), and pertussis toxin (Sigma) were performed for 15 min before IFN-γ or eotaxin stimulation for 1 h at 37 °C. Inhibitors were diluted in DMSO at a final concentration $<0.01\%$, which had no effect on granule secretion. TEM studies were performed on granules treated with and without BFA for 15 min before IFN-γ addition.

Granule Isolation Following Eosinophil Cytolytic Degranulation. Plasma-coated beads were made as previously described (32) with modifications. Briefly, Sepharose 6B beads (Amersham-Amersham Pharmacia) were incubated with 10% human plasma plus 4.5 μg/ml cobra venom factor (Complement Technologies) for 15 min at 37 °C. Eosinophils (5×10^6 eosinophils per 1.2 ml) were mixed with 500 μl plasma-coated beads and incubated for 50 min at 37 °C. Granules released by cytolysis (19, 32) were recovered in supernatants.

Assays of Eosinophil Granule and Granule-Secreted Proteins. EPO activity was measured by a colorimetric assay (33) in subcellular fractions and in eosinophil granule supernatants. For β-hex activity, 50-μl samples were incubated with 50 μl substrate solution [1 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma) in 0.2 M citrate buffer, pH 4.5, plus 0.1% Triton X-100] at 37 °C for 2 h. Reactions were terminated by addition of 100 μl of 1 M Tris (pH 8), and fluorescence (excitation, 360 nm; emission, 460 nm) was measured in a CytoFluor 2350 plate reader (Millipore).

ECP levels in supernatants of eosinophil granules and sonicated- or cell extraction buffer– (FNN0011; Biosource) disrupted granules were quantified by ECP ELISA kits (Medical & Biological Labs). Stimulated ECP secretion represents ECP levels from stimulated samples minus ECP levels from unstimulated samples.

Cytokines, IL-4, IL-8, IL-6, IL-10, IFN-γ, IL-13, and IL-12 (*p*-70) were quantified using multiplex assays (Bio-Rad Laboratories, Inc.).

Flow Cytometry of Eosinophil Granules. Isolated granules were incubated either with primary FITC-conjugated Ab (45 min) or primary (1 h) and then FITC-conjugated secondary (15 min) Abs on ice in the absence of granule fixation. After staining, granules were fixed in buffer containing 2% paraformaldehyde without methanol (Electron Microscopy Sciences) for 5 min. For intragranular staining, isolated granules were fixed for 5 min in 2% paraformaldehyde and permeabilized for 5 min on ice with 0.1% saponin before incubation with primary (1 h) and secondary (15 min) Abs. Control or nonimmune Abs were included for all. Analyses were performed on a FACScan with CELLQUEST software (BD Biosciences).

For nonpermeabilized granules, rabbit anti-human LAMP-2 Ab (H-207, 5 μg/ml; Santa Cruz Biotechnology) and mouse anti-human CD63 (LAMP-3) mAb (clone H5C6, 20 μg/ml; BD PharMingen) were used in parallel with respective control nonimmune IgGs. Anti-rabbit and anti-mouse FITC-conjugated Abs, respectively, were secondary Abs (1:100; Jackson ImmunoResearch). Hamster anti-human IFN-$\gamma\alpha$R (clone 2E2, 5 μg/ml; Santa Cruz Biotechnology), rat anti-human CCR3 (clone 61828.11, 5 μg/ml; R&D), and mouse anti-human MHC class I (HLA-ABC, clone G46–2.6, 14 μg/ml; BD PharMingen) FITC-conjugated mAbs, each directed at extracellular domains, were used with FITC-conjugated IgG control mAbs. Goat Ab generated against a peptide in the intracellular carboxyl terminus of human CCR3 (5 μg/ml, C20; Santa Cruz Biotechnology) was used with a control nonimmune goat IgG and anti-goat FITC-conjugated secondary Ab (1:100; Jackson ImmunoResearch). On permeabilized granules, mouse anti-human MBP mAb (5 μg/ml, clone AHE-2; BD PharMingen) and an isotype mAb were used, and the secondary Ab was an FITC-conjugated goat anti-mouse Ab (1:100; Jackson ImmunoResearch).

Western Blotting. Granules were lysed in a 0.5% hexadecyl trimethylammonium bromide (Sigma) Tris buffer (10 mM, pH 7.4) containing 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and protease inhibitors mixture (P8340, 1:100; Sigma). For phosphorylation assays, we added phosphatase inhibitor cocktails 1 and 2 (P2850 and P5726, each at 1:100; Sigma). Pools of lysed granules from three subcellular fractionations were loaded on 10% Bis-Tris gels (Invitrogen) under denaturing conditions. Gels were transferred to nitrocellulose membranes (Pierce), blocked overnight with 5% BSA, and probed with rabbit anti-phospho-p38 MAPK pAb that detects dually phosphorylated Thr-180 and Tyr-182 (1:200; Cell Signaling), rabbit anti-human PKC α,β,γ pAb (1:200; Abcam), and rabbit anti-human *p*-38 MAPK $\alpha,\beta,\gamma,\sigma$ pAb (1:500; Cell Signaling), followed by anti-rabbit secondary Ab conjugated to HRP (1:15,000; Jackson ImmunoResearch). A mouse anti-human MBP mAb (1:200, clone AHE-2; BD PharMingen) and a mouse anti-phosphotyrosine mAb (1:1,000, clone 4G10; Upstate) were used to detect MBP and tyrosine phosphorylated residues, respectively, followed by anti-mouse secondary Ab conjugated to HRP (1:15,000; Jackson ImmunoResearch). Membranes were developed with West Fento chemiluminescence kits (Pierce).

Activation of AO-Bearing Eosinophil Granules. AO labels acidic lysosomal eosinophil granules (34). As previously described for a mast cell line, activation of granules leads to the release of monomeric AO at a neutral pH, which exhibits a spectral shift to green yielding transient green flashes from responding granules (18). To help ascertain that granules obtained by subcellular fractionation and by eosinophil cytolysis were the organelles responsive to IFN-γ and eotaxin agonists, we monitored the responses of AO-labeled eosinophil granules by fluorescence microscopy. Cell-free granules were loaded with 3 μM AO (10 min, 4 °C) (Sigma), washed, and resuspended in Ca^{2+}/Mg^{2+} containing HBSS ($HBSS^{++}$). Granules were spread on polyL-lysine–coated slides (5 μl per slide) and coverslipped. Stimuli in stock solutions of IFN-γ (700 ng/ml) or eotaxin (350 ng/ml) or control $HBSS^{++}$ was introduced in 2-μl drops at the edge of the coverslips, enabling stimuli to diffuse under the coverslip. Responses of AO-stained granules were monitored by fluorescence microscopy using the FITC band fluorescence filter. To prevent AO quenching during time-lapse microscopy, three neutral density filters (ND6, ND25, and ND12) were used during data acquisition. To compensate for the low fluorescence signal, the gain of the camera was increased to 100 before each recording. Fluorescence images were recorded for 5 min (100 frames in total) and were acquired using a Hamamatsu Orca AG cooled monchrome CCD camera (Bridgewater) coupled to a BX-62 Olympus microscope (Olympus) using a UplanApo objective (40×1.35). The microscope, Z-motor drive, shutters, and camera were controlled by IVision 4.0 for Mac (BioVision Technology). Acquired frames were further processed into movies with IVision. Images were pseudocolored to display intensities in AO fluorescence.

TEM of Tissue and Granule Samples. A lesional skin biopsy was obtained with informed consent from a subject with a hypereosinophilic syndrome, as approved by the Committee on Clinical Investigation. Granule samples in agar and skin tissue were prepared for TEM as before (14, 15). Ten granule samples from different experiments were analyzed by TEM to ensure granule purity. For identification and quantification of lipid deposits in BFA- and control-treated granules, a total of 145 electron micrographs were randomly taken at $\times$21,000 and analyzed at a final magnification of $\times$58,000. Between 419 and 529 granules per condition were evaluated blindly by two observers to enumerate membranolipid deposits within BFA- and control-treated granules.

Statistical Analysis. Data were expressed as means of replicates $\pm$ SD. Results were analyzed by one-way ANOVA, followed by the Newman-Keuls test. *P* values <0.05 were considered significant.

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